

Binding and Accumulation of Hemin in *Porphyromonas gingivalis* Are Induced by Hemin

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Although hemin is an essential nutrient for the black-pigmented oral bacterium *Porphyromonas gingivalis*, the mechanisms involved in hemin binding and uptake are poorly defined. In this study, we have examined the binding of hemin and Congo red (CR) to *P. gingivalis* whole cells and have defined the conditions for maximal binding. Additionally, the accumulation of hemin by *P. gingivalis* under growing conditions has been characterized. *P. gingivalis* A7436 was grown under hemin- or iron-deplete conditions (basal medium [BM] or Schaedler broth with dipyriddy [SBD]) or under hemin- or iron-replete conditions (BM with hemin [BMH] or Schaedler broth [SB]), and hemin and CR binding were assessed spectrophotometrically. Binding of hemin by *P. gingivalis* whole cells was rapid and was observed in samples obtained from cells grown under hemin- and iron-replete and hemin-deplete conditions but was not observed in cells grown under iron limitation. We also found that *P. gingivalis* whole cells bound more hemin when grown in BMH or SB than cells grown in BM or SBD. Binding of CR by *P. gingivalis* A7436 was also enhanced when cells were grown in the presence of hemin or when cells were incubated with hemin prior to CR binding. Hemin binding and accumulation were also assessed using [¹⁴C]hemin and [⁵⁹Fe]hemin under growing conditions. Both [¹⁴C]hemin and [⁵⁹Fe]hemin were accumulated by *P. gingivalis*, indicating that iron and the porphyrin ring were taken into the cell. Binding and accumulation of hemin under growing conditions were also induced by growth of *P. gingivalis* in hemin-replete media. Hemin accumulation was inhibited by the addition of KCN to *P. gingivalis* cultures, indicating that active transport was required for hemin uptake. [¹⁴C]hemin binding and accumulation were also inhibited by the addition of either cold hemin or protoporphyrin IX. Taken together, these results indicate that *P. gingivalis* transports the entire hemin moiety into the cell and that the binding and accumulation of hemin are induced by growth of cultures in the presence of hemin.

The black-pigmented obligate anaerobe *Porphyromonas (Bacteroides) gingivalis* has been implicated as a major pathogen in destructive periodontal disease (53). As with other pathogens, a requirement for the in vivo growth of *P. gingivalis* is that the organism must be capable of obtaining iron from the host. The low concentration of free iron in body fluids creates bacteriostatic conditions for many microorganisms and is an important defense factor against invading bacteria (6, 64). Within the human host, potential iron sources available to pathogenic bacteria include transferrin found in serum and lactoferrin present on mucosal surfaces (39). In addition, heme-containing compounds are a particularly abundant source of in vivo iron. Pathogens which occupy intracellular niches in vivo can utilize heme directly. However, extracellular pathogenic bacteria can utilize the iron in heme compounds only after the heme is released; this typically occurs by some form of tissue damage resulting in the release of intracellular material (39). The ability to utilize hemin and hemin-containing compounds for nutritional iron has been documented for several pathogenic bacteria, including *Vibrio cholerae*, *Vibrio vulnificus*, *Klebsiella pneumoniae*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Yersinia pestis*, *Yersinia enterocolitica*, *Shigella flexneri*, *Bacteroides fragilis*, *Bordetella pertussis*, *Streptococcus pneumoniae*, *Haemophilus ducreyi*, and *Haemophilus influenzae* (10, 14, 16, 21–23, 29, 30, 39, 44, 46, 56, 58, 60, 62, 63). *P. gingivalis* also utilizes iron in the form of hemin for growth (20,

31); however, the mechanisms utilized for hemin transport are poorly understood.

P. gingivalis is capable of utilizing in vitro a broad range of hemin-containing compounds such as hemoglobin, myoglobin, hemopexin, methemoglobin, oxyhemoglobin, and cytochrome *c* (1, 2). *P. gingivalis* can also grow with nonhemin iron sources, including ferric and ferrous inorganic iron, and human transferrin (2, 25). Protoporphyrin IX, the Fe-free precursor of hemin, has also been shown to support the growth of *P. gingivalis* when at least 10⁻⁷ M iron was present (66). Growth of *P. gingivalis* with hemin determines doubling time, cell yield, content, and proteolytic activity and also appears to influence the pathogenic potential of certain strains (7, 33, 35, 37, 49, 55). The characteristic pigmentation produced by *P. gingivalis* colonies appears to be due to the accumulation of hemin on the cell surface and has been postulated to serve as a mechanism for hemin storage (52, 54). Hemin can satisfy the entire iron requirement of *P. gingivalis* as well as provide it with the ability to utilize endogenous membrane-stored hemin for growth during periods of hemin deprivation. The ability of *P. gingivalis* to store hemin appears to provide a nutritional advantage for the survival of this pathogen in the iron-limited environment of the healthy periodontal pocket. The environmental conditions in the periodontal pocket during the course of an infection are not precisely known; however, high numbers of erythrocytes are typically found in diseased sites (32, 38). A recently described *P. gingivalis* hemolysin, whose activity is regulated by environmental hemin, may function to lyse erythrocytes in vivo, resulting in the liberation of hemin (9). *P. gingivalis* also appears to be highly effective at degrading the plasma proteins albumin, hemopexin, haptoglobin, and TF (35). The production of these proteolytic moieties by *P.*

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gingivalis has been postulated to contribute to the degradation of host heme-sequestering proteins. Precisely how the heme molecule is taken into the *P. gingivalis* cell and processed is not known.

The aromatic sulfonated diazo dye Congo red (CR) has been used to examine the mechanisms of heme binding and assimilation in *B. fragilis*, *Y. pestis*, *Y. enterocolitica*, *Aeromonas salmonicida*, *Actinobacillus pleuropneumoniae*, *B. pertussis*, and *S. flexneri* (12, 13, 27–29, 34, 40, 47, 50, 61). CR binding to the cell surface is proposed to induce the same cell surface binding receptor employed for heme binding in these species and is stimulated under conditions of iron limitation. In this study, we have examined the binding of heme and CR by *P. gingivalis* whole cells under nongrowing conditions and have determined the relationship between CR and heme binding. Additionally, we have utilized [¹⁴C]heme and [⁵⁹Fe]heme to examine the ability of *P. gingivalis* to bind and accumulate heme into the cell under growing conditions.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. gingivalis* A7436, a clinical isolate originally characterized by V. R. Dowell (Anaerobic Microbiology Laboratory, Centers for Disease Control, Atlanta, Ga.) (17, 18), was used in these studies. *P. gingivalis* cultures were typically maintained on anaerobic blood agar (Remel, Lenexa, Kans.) at 37°C in an anaerobe chamber (Coy Laboratory Products Inc.) with 85% N₂, 5% H₂, and 10% CO₂. After incubation at 37°C for 3 days, cultures were inoculated into basal medium (BM [containing the following in grams per liter: trypticase peptone, 10; tryptophan, 0.2; NaCl, 2.5; sodium sulfite, 0.1; and cysteine, 0.4]) and incubated at 37°C under anaerobic conditions for 24 h. This culture served as the inoculum into BM, BM supplemented with heme (1.5 μM) (BMH), Schaedler broth (SB) (Difco Laboratories), or SB plus dipyrindyl (20 μg/ml) (SBD). Growth was monitored as the A₆₆₀ (Beckman DU8 spectrophotometer).

The iron content of BM and SB was determined by the ferrozine assay (58) or by mass spectrophotometry. Analysis of iron content indicated that SB contained 10.9 μM iron and that BM contained 2.6 μM iron.

Heme and CR binding assays. The binding of heme and CR to *P. gingivalis* whole cells under nongrowing conditions was measured by a modification of the procedure described by Deneer and Potter (13). Our previous studies have indicated that saturation of heme and CR binding by *Bacteroides thetaiotaomicron* occurs at 20 and at 30 μg/ml, respectively (19). *P. gingivalis* cultures were grown in BM, BMH, SB, or SBD for 24 h, harvested by centrifugation at 8,000 × g for 10 min, washed in phosphate-buffered saline (PBS), and resuspended in PBS to an optical density at 660 nm (OD₆₆₀) of 1.5 (10¹⁰ CFU/ml). To this was added CR or heme to a final concentration of 30 μg/ml. A 1.0-ml sample was immediately removed and centrifuged for 1 min at 10,000 × g in an Eppendorf centrifuge to pellet the cells, and the supernatant was assayed spectrophotometrically for heme (OD₄₀₀) or CR (OD₄₈₈). The remaining cells were incubated at 37°C under anaerobic conditions and assayed at 5- to 15-min intervals for residual heme or CR in the supernatant. *P. gingivalis* cultures without added heme or CR, or heme or CR without added cells, served as controls. The concentration of heme or CR present in the supernatant fractions was calculated from a heme or CR standard curve. Binding of heme or CR to whole cells was calculated as the difference between the total amount

of heme or CR added versus the amount remaining in the supernatant at each time point compared with time zero.

For competition assays, heme (30 μg/ml) was allowed to prebind to *P. gingivalis* whole cells (in PBS) for 60 min at 37°C under anaerobic conditions. Excess heme was removed by washing with PBS. Following prebinding, CR (30 μg/ml) was added, and binding was determined as described above. In a separate experiment, we also examined the potential competition between heme and CR during the CR binding assay. For these experiments, heme and CR (30 μg/ml) were added simultaneously to whole cells at time zero, which was followed by incubation as described above. Binding of CR to whole cells was calculated from a standard curve as described above.

Binding and accumulation of radiolabelled heme. [¹⁴C]heme (University of Leeds Innovations, Ltd., Leeds, England) and [⁵⁹Fe]heme (Dupont Co., Wilmington, Del.) were used in studies to examine the accumulation of heme and the iron from heme by *P. gingivalis*. All glassware was washed with nitric acid (10%) and thoroughly rinsed in deionized water before use. *P. gingivalis* cultures were grown in BM or BMH to the exponential phase of growth, and each culture was divided into two separate flasks. Each culture was then washed in PBS and resuspended in BM to an OD₆₆₀ of 0.10. To one flask of cells grown in BM and one flask of cells grown in BMH was added 20 μM KCN and cells incubated at 37°C under anaerobic conditions for 1 h. [¹⁴C]heme (1.5 or 4.0 μM; specific activity, 112 Ci/mol) or [⁵⁹Fe]heme (50 μM; specific activity, 1.5 Ci/mol) was then added to all cultures, and the cultures were incubated for an additional 24 h at 37°C under anaerobic conditions. Duplicate samples of cells were removed from each flask at 0, 1, 4, 8, and 24 h, diluted into 10 ml of 0.1 M sodium citrate buffer (pH 7.4) containing 1.0 μM MgCl₂ and 0.25 mM CaCl₂, and filtered through 0.45-μm-pore size cellulose acetate filters. The filters were washed twice with 3 ml of 0.02 N NaOH in 50% ethanol and air dried overnight, and the amount of cell-associated [¹⁴C]heme or [⁵⁹Fe]heme was determined by liquid scintillation spectrometry. Cell-associated [¹⁴C]heme and [⁵⁹Fe]heme are expressed in picomoles per milligram of total cellular protein. Total protein content was determined for each sample by use of a bicinchoninic acid protein assay with bovine serum albumin as the standard (Pierce, Rockford, Ill.). Heme uptake was calculated as the difference between the [¹⁴C]heme or [⁵⁹Fe]heme associated with untreated cultures versus KCN-treated cultures and is expressed as picomoles per milligram of total cellular protein for each time point.

Competition assays. For competition assays, unlabeled heme (1.5 μM) or protoporphyrin IX (1.5 μM) was added to *P. gingivalis* cultures following KCN treatment as described above. [¹⁴C]heme (1.5 μM) was then added, and the uptake of radiolabelled heme was determined as described above. Competition was determined by comparing [¹⁴C]heme binding and accumulation of cultures containing cold heme or protoporphyrin IX to cultures that contained only [¹⁴C]heme.

RESULTS

Effect of heme and iron restriction on growth of *P. gingivalis*. When *P. gingivalis* A7436 was grown in BMH and subsequently transferred to heme-free BM, the culture continued to grow for several generations (data not shown). These cells apparently had accumulated heme during growth in heme-sufficient media and utilized this endogenous pool until exhausted. However, when *P. gingivalis* was initially grown in BM for 24 h and then reinoculated into fresh BM, minimal growth was observed (Fig. 1). Therefore, for all assays de-

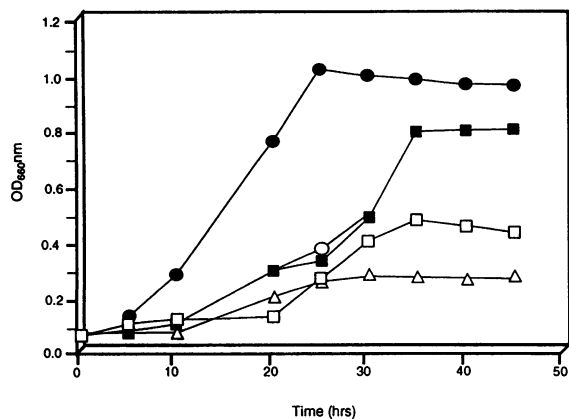


FIG. 1. Growth of *P. gingivalis* A7436. *P. gingivalis* was initially grown in BM for 24 h and then reinoculated into BM (Δ), BM plus protoporphyrin IX (\square), BMH (\circ), SB (\bullet), and SBD (\blacksquare). Results are from one experiment and are representative of three separate experiments.

scribed below, *P. gingivalis* was initially grown in BM to exhaust stored hemin pools.

Growth of *P. gingivalis* A7436 in SB resulted in a typical growth curve with a final cell density of 10^{10} CFU (Fig. 1 and data not shown). Addition of the ferrous iron chelator dipyriddy (20.0 $\mu\text{g}/\text{ml}$) to SB effectively suppressed growth of *P. gingivalis* (Fig. 1). Increasing concentrations of dipyriddy completely arrested *P. gingivalis* growth (data not shown). *P. gingivalis* grown in BM supplemented with hemin exhibited a typical growth curve, although the final cell density of 10^8 CFU/ml was lower than that of cultures grown in SB (Fig. 1 and data not shown). In agreement with previous studies (1, 65), we found that *P. gingivalis* grew with protoporphyrin IX as a substitute for hemin; similar growth curves were obtained with hemin or protoporphyrin IX supplementation (Fig. 1). These results indicate that protoporphyrin IX can serve as a porphyrin source for *P. gingivalis* and also suggest that *P. gingivalis* may acquire sufficient iron from the small amount present in BM.

Hemin binding assay. To examine the influence of hemin and/or iron on hemin binding by *P. gingivalis*, we examined hemin binding by a spectrophotometric assay. *P. gingivalis* cultures grown in hemin-replete (BMH) or hemin-deplete (BM) medium were found to bind hemin quickly, as detected within the first 10 min (Fig. 2A). Interestingly, hemin binding by *P. gingivalis* whole cells was enhanced when cultures were grown under hemin-replete conditions. *P. gingivalis* grown in BMH bound 22% more hemin (a statistically significant increase [$P < 0.05$]) than *P. gingivalis* grown in BM as detected at 10 min (Fig. 2A). This represented 8% of the total hemin present. Cultures of *P. gingivalis* grown in iron-replete conditions (SB) also bound hemin quickly, as detected within the first 10 min (Fig. 2B). *P. gingivalis* grown in SB bound 2.52 μg of hemin, as detected at 20 min (Fig. 2B). However, cultures of *P. gingivalis* A7436 grown under iron-restricted conditions (SBD) bound minimal amounts of hemin (0.55 to 0.75 μg), a statistically significant decrease ($P < 0.05$) (Fig. 2B). Taken together, these results indicate that growth of *P. gingivalis* under hemin- and iron-replete conditions enhances hemin binding by *P. gingivalis* whole cells under nongrowing conditions.

CR binding assay. In *A. pleuropneumoniae*, *S. flexneri*, and *A.*

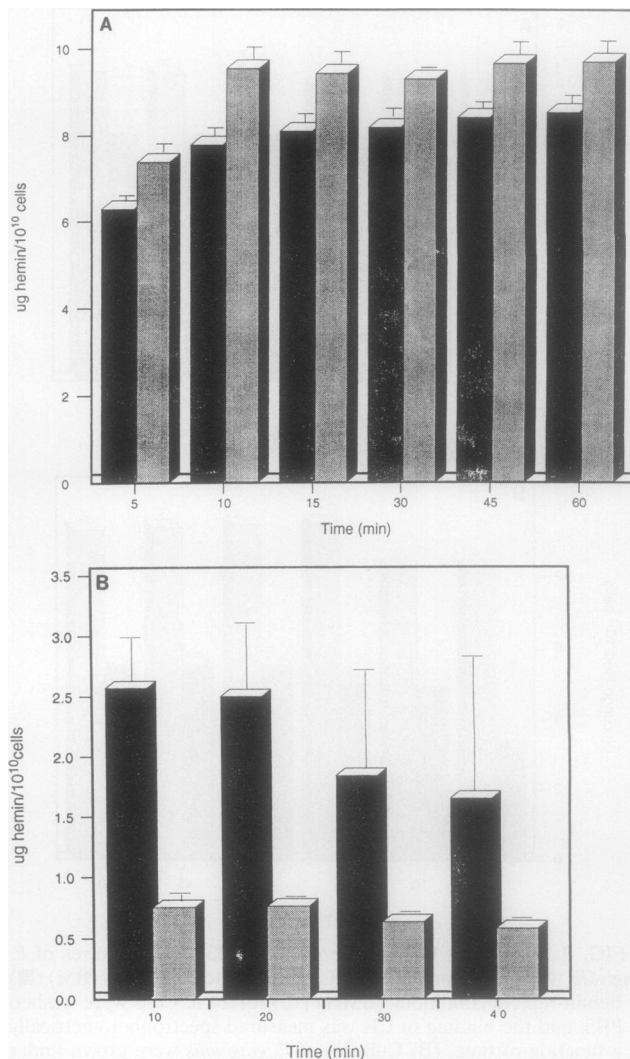


FIG. 2. Binding of hemin by *P. gingivalis* A7436. (A) Cultures of *P. gingivalis* were grown under either hemin-deplete conditions (BM) (\blacksquare) or hemin-replete conditions (BMH) (\blacksquare), and the binding of hemin was measured spectrophotometrically as a function of time as described in Materials and Methods. (B) Cultures of *P. gingivalis* were grown under either iron-deplete conditions (SBD) (\blacksquare) or iron-replete conditions (SB) (\blacksquare), and the binding of hemin was measured spectrophotometrically as a function of time as described in Materials and Methods. Results are presented as the means \pm standard deviations of two or three separate experiments.

salmonicida, it has been suggested that CR binding is related to the ability of these organisms to sequester iron (13, 27, 59). A strong correlation exists between CR binding and hemin adsorption, and Cr binding is stimulated under conditions of iron limitation. To determine if a similar relationship exists in *P. gingivalis*, we examined the CR binding abilities of whole cells obtained from cultures grown under hemin or iron restriction. In agreement with the results obtained for hemin binding, *P. gingivalis* whole cells bound more CR when cultures were grown in media supplemented with hemin ($P < 0.05$ for all time points) (Fig. 3A). Whole cells obtained from cultures grown in BM bound 7.6 μg of CR (15-min sample); in contrast, cells obtained from cultures grown in BMH bound 10.0 μg of CR. Preincubation of whole cells of *P. gingivalis* grown in BM

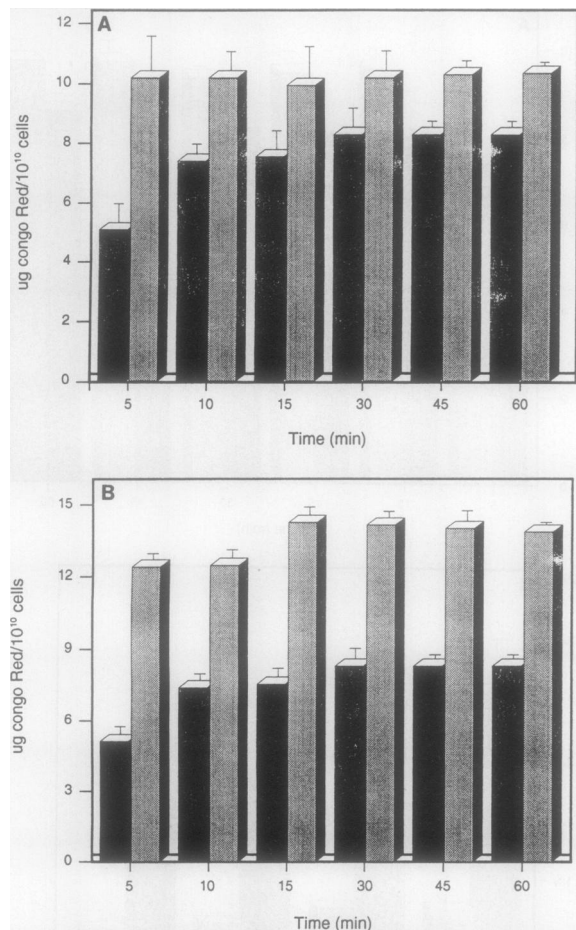


FIG. 3. Binding of CR by *P. gingivalis* A7436. (A) Cultures of *P. gingivalis* were grown under either hemin-deplete conditions (BM) (■) or hemin-replete conditions (BMH) (▨) for 24 h. Cells were washed in PBS, and the binding of CR was measured spectrophotometrically as a function of time. (B) Cultures of *P. gingivalis* were grown under hemin-deplete conditions (BM). Cells were harvested and washed in PBS, and hemin (30 μ g/ml) was allowed to prebind onto whole cells. Excess hemin was removed by washing with PBS, which was followed by the addition of CR (30 μ g/ml). ▨, hemin prebind; ■, no prebind. Results are presented as the means \pm standard deviations of two separate experiments.

with hemin (30 μ g/ml) prior to CR addition also resulted in an increased binding of CR (Fig. 3B). Whole cells preincubated with hemin bound 12.4 μ g of CR (as detected at 5 min), compared with 5.1 μ g bound by control cultures. CR binding was also increased when hemin was present in the CR assay (data not shown). When protoporphyrin IX was allowed to prebind *P. gingivalis* whole cells (grown in BM), CR binding was also increased (data not shown). These results indicate that growth of *P. gingivalis* in hemin-containing media or preincubation of whole cells with hemin or protoporphyrin IX under nongrowing conditions results in increased Cr binding.

Hemin binding under growing conditions with [14 C]hemin.

To examine the kinetics of hemin binding under growing conditions, we also determined *P. gingivalis* hemin binding using [14 C]hemin as the sole hemin source during growth in BM. For these studies, cultures of *P. gingivalis* A7436 were grown under either hemin-deplete (BM) or hemin-replete (BMH) conditions for 24 h and reinoculated into fresh BM.

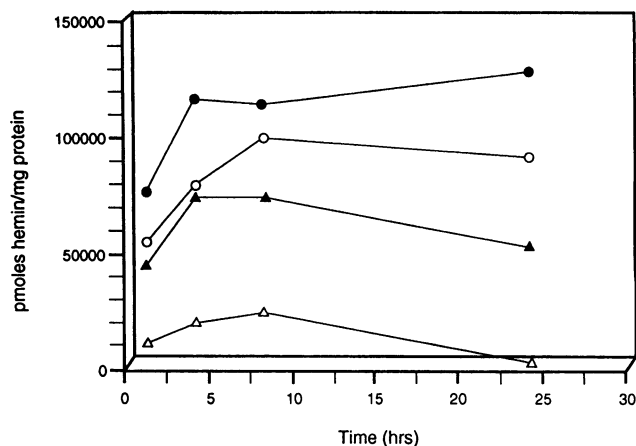


FIG. 4. Hemin binding and accumulation by *P. gingivalis* A7436. Cultures of *P. gingivalis* A7436 were grown under either hemin-deplete conditions (BM) (○ and △) or hemin-replete conditions (BMH) (● and ▲) for 24 h. Cells were suspended in fresh BM supplemented with [14 C]hemin, and hemin binding and uptake were determined under growing conditions with [14 C]hemin (4.0 μ M) as a function of time as described in Materials and Methods. The amount of hemin bound was determined from the total hemin associated with whole cells and is expressed as picomoles per milligram of total cellular protein for each time point. ●, hemin binding of BMH cultures; ○, hemin binding of BM cultures. Hemin uptake was calculated as the difference between the [14 C]hemin associated with untreated versus KCN treated cultures and is expressed in picomoles per milligram of total cellular protein for each time point. Results are from one experiment and are representative of four separate experiments. ▲, hemin uptake of BMH cultures; △, hemin uptake of BM cultures.

Binding was determined under growing conditions by supplementation of cultures with [14 C]hemin (4.0 μ M), and hemin binding was expressed as the total hemin associated with whole cells. In agreement with the results obtained under nongrowing conditions, *P. gingivalis* bound more hemin when cells were initially grown under hemin-replete conditions (BMH) (Fig. 4). Hemin binding was initially detected at 30 min (data not shown), and the amount of hemin bound by *P. gingivalis* increased over time, with maximal binding by 24 h (Fig. 4). *P. gingivalis* grown in BMH bound 115,000 pmol/mg of protein, 32% more hemin than *P. gingivalis* grown in BM, as detected at 4 h. At 24 h, *P. gingivalis* grown in BMH bound 29% more hemin than *P. gingivalis* grown in BM.

Hemin accumulation. The time course of uptake of hemin during logarithmic growth of *P. gingivalis* is shown in Fig. 4. To confirm the active transport of hemin, KCN, an inhibitor of energy transduction (10), was used. Minimal hemin was associated with *P. gingivalis* whole cells following KCN treatment, indicating that energy was required for uptake. The accumulation of hemin was calculated as the difference between the [14 C]hemin associated with untreated cells (total hemin) versus KCN treated cells and is expressed in picomoles per milligram of total cellular protein for each time point. Hemin was taken up by *P. gingivalis* quickly (initially detected at 30 min; data not shown); cultures of *P. gingivalis* initially grown in BMH and sampled at 1 h accumulated 43,000 pmol of hemin per mg of protein (Fig. 4). In agreement with the results obtained for hemin binding under nongrowing and growing conditions, more hemin was accumulated by *P. gingivalis* when cultures were initially grown with hemin (Fig. 4). *P. gingivalis* initially grown in BMH accumulated 73,000 pmol of hemin per mg of protein as detected at 4 h. This time point is equivalent to

approximately one generation, as determined by measurements of OD and CFU (data not shown). Cultures exhibited typical growth, as determined by viable counts and protein concentration, indicating that radiolabelled hemin was sufficient to support bacterial growth (data not shown). Accumulation of hemin continued steadily and leveled off at 24 h when the cultures reached stationary phase. At this point, hemin uptake decreased relative to that of samples removed at early log phase. These results indicate that as with hemin binding, in *P. gingivalis* hemin accumulation in growing cells is also regulated by intracellular hemin.

Competition assay. To determine if protoporphyrin IX or hemin could compete with radiolabelled hemin for binding and uptake and to confirm the specific accumulation of hemin, we examined the uptake of radiolabelled hemin in the presence of cold hemin or protoporphyrin IX. Both protoporphyrin IX and cold hemin were shown to compete for hemin binding and accumulation (Fig. 5A and B). Cultures of *P. gingivalis* to which 1.5 μ M cold hemin had been added bound 28,000 pmol/mg of hemin, a 30% reduction in hemin bound compared with that of control cultures sampled at 4 h (Fig. 5A). Likewise, hemin accumulation by these cultures was 5,000 pmol/mg, a 61% reduction in hemin accumulation (Fig. 5B). The addition of protoporphyrin IX resulted in a 28% inhibition of hemin binding and a 46% inhibition of hemin accumulation as detected at 4 h (Fig. 5A and B). When *P. gingivalis* was initially grown under hemin-deplete conditions and similar competition assays were performed, hemin binding and accumulation were also decreased (data not shown).

Accumulation of Fe from hemin by *P. gingivalis*. The results obtained using [14 C]hemin indicated that *P. gingivalis* can take up the entire hemin molecule into the cell. To examine the ability of *P. gingivalis* to accumulate the iron from hemin into the cell, we examined hemin accumulation using [59 Fe]hemin. As seen in Fig. 6, our results indicate that like the protoporphyrin ring, the Fe from hemin was also accumulated into *P. gingivalis* quickly. Cultures initially grown in BMH and sampled at 1 h accumulated 950 pmol of [59 Fe]hemin per mg of protein (Fig. 6). In agreement with results obtained using [14 C]hemin, we also found that more [59 Fe]hemin was taken up by *P. gingivalis* when cultures were initially grown with hemin. *P. gingivalis* grown in BMH accumulated 920 pmol of hemin per mg of protein as detected at 4 h. In contrast, *P. gingivalis* grown in BM accumulated only 143 pmol of hemin per mg of protein as detected at 4 h, a statistically significant decrease ($P < 0.05$).

DISCUSSION

A number of gram-negative bacterial pathogens can utilize hemin as a source of iron (39), and, of these, a few can also utilize hemin as a porphyrin source. For example, *Escherichia coli*, *Y. pestis*, and *Salmonella typhimurium* are normally unable to utilize hemin as a porphyrin source since their outer membranes are impermeable to hemin (36, 51). By contrast, *Y. enterocolitica*, *S. flexneri*, and *H. influenzae* can utilize hemin as both an iron and porphyrin source since hemin can permeate the outer membrane (10, 57). In the present study, we have demonstrated that, like *Y. enterocolitica* and *H. influenzae*, *P. gingivalis* is capable of transporting the entire hemin molecule into the cell. Additionally, we found that binding of hemin to *P. gingivalis* whole cells under nongrowing conditions and binding and accumulation of hemin under growing conditions are induced by growth of *P. gingivalis* in hemin-replete conditions. We have also demonstrated that CR binding by *P. gingivalis* whole cells is hemin inducible. This was observed

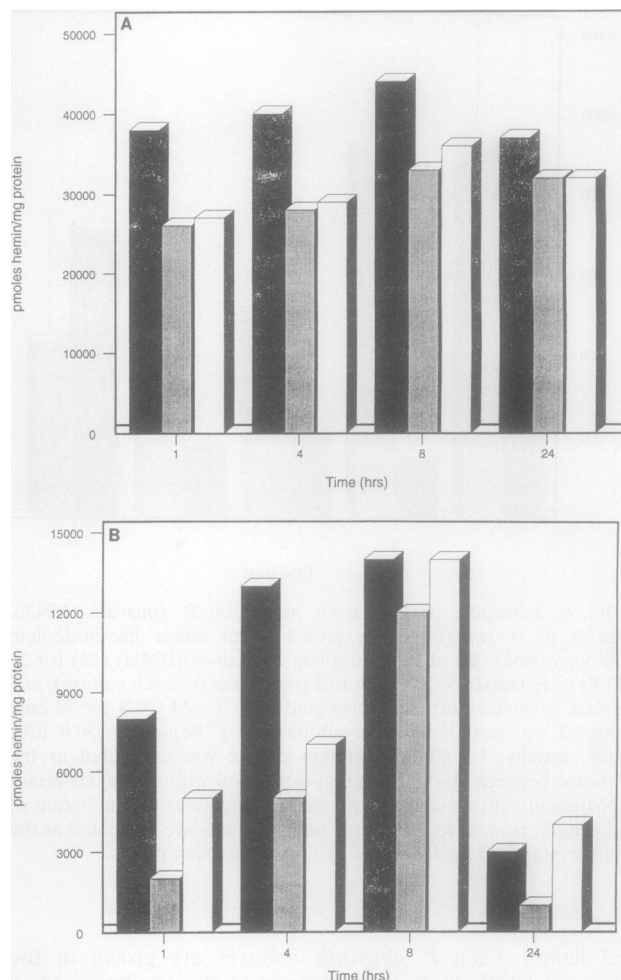


FIG. 5. Binding and accumulation of radiolabelled hemin in the presence of cold hemin and protoporphyrin IX by *P. gingivalis* A7436. Cultures of *P. gingivalis* were grown under hemin-replete conditions (BMH) for 24 h. Cells were suspended in fresh BM (two flasks), and one flask was incubated with 20 μ M KCN for 30 min. Unlabeled hemin (12.5 μ M) or protoporphyrin IX (1.5 μ M) was added to KCN-treated and untreated *P. gingivalis* cultures, and then [14 C]hemin was added (1.5 μ M; specific activity, 112 Ci/mol). Hemin uptake was calculated as the difference between the [14 C]hemin associated with untreated versus KCN-treated cultures and is expressed in picomoles per milligram of total cellular protein for each time point. ■, unlabeled hemin; ▒, unlabeled protoporphyrin IX; □, control. Results are from one experiment and are representative of two separate experiments. (A) Total hemin bound; (B) hemin accumulation.

when cells were grown in the presence of hemin or when hemin was allowed to prebind whole cells prior to CR binding. Taken together, these results indicate that hemin and CR binding in *P. gingivalis* may be very closely linked and that a hemin-inducible component must be activated for binding to occur.

Bramanti and Holt (3, 4) have described a 26-kDa protein that is produced by *P. gingivalis* W50 grown under conditions of hemin limitation and have proposed a role for this protein in hemin binding and transport. This newly discovered protein appears to be associated primarily with the outer membrane. These authors have proposed its involvement in the outer membrane for hemin binding followed by importation across the outer membrane for intracellular hemin transport. Bramanti and Holt (5) recently reported that this Omp appears to

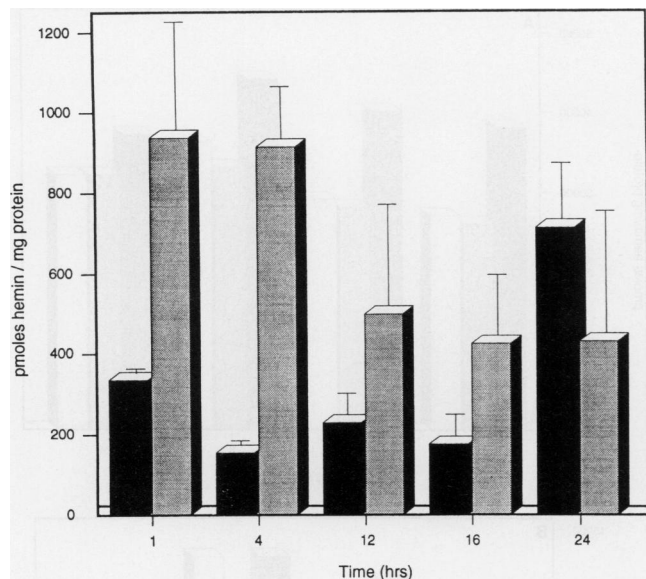


FIG. 6. Transport of iron from hemin by *P. gingivalis* A7436. Cultures of *P. gingivalis* were grown under either hemin-deplete conditions (BM) (■) or hemin-replete conditions (BMH) (▨) for 24 h. Cells were suspended in fresh BM (two flasks for each culture), and one flask of each culture was incubated with 20 μ M KCN for 30 min. Transport was initiated by the addition of [59 Fe]hemin (50.0 μ M; specific activity, 1.5 Ci/mol). Hemin uptake was calculated as the difference between the [59 Fe]hemin associated with untreated versus KCN-treated cultures and is expressed in picomoles per milligram of total cellular protein for each time point. Results are presented as the means \pm standard deviations of two separate experiments.

bind hemin when *P. gingivalis* cultures are grown in the presence of [59 Fe]hemin. In contrast to the results reported here, these investigators found that the uptake of [59 Fe]hemin by *P. gingivalis* was induced under conditions of hemin starvation or by treatment with the iron chelator 2,2'-dipyridyl. The addition of antisera to the Omp26 to hemin-starved cells was found to inhibit [59 Fe]hemin uptake, implicating the Omp26 protein in hemin transport. The discrepancy in results presented here with those presented by Bramanti and Holt may be due to several factors. For the studies described here, a minimal medium was used; in contrast, Bramanti and Holt (5) employed a complex medium for their hemin uptake studies. In addition, in the present study, we examined the uptake of hemin throughout the entire growth cycle (24 h), whereas in the studies described by Bramanti and Holt (5) hemin uptake was examined only to 50 min. Finally, in this study, we found that hemin induced hemin binding both under growing and nongrowing conditions, as assessed in two different assays.

In addition to the production of hemin-repressible proteins, additional *P. gingivalis* iron-repressible outer membrane proteins have been observed (1, 8). Interestingly, we observed the production of novel proteins which are apparently induced in the presence of hemin (19a). The induction of novel proteins and of hemin binding in response to hemin may represent a novel mechanism for the acquisition of hemin by *P. gingivalis*. We have recently isolated and characterized a nonpigmented transpositional insertion mutant of *P. gingivalis* (designated MSM-3) that binds less hemin than the wild-type pigmented strain, irrespective of the presence of hemin in the growth medium (19a). Internalization of [14 C]hemin by *P. gingivalis* MSM-3 is also decreased relative to that of the parent strain,

consistent with the inability of this strain to grow efficiently with hemin as a sole source of iron. Further characterization of the genetic lesion in MSM-3 should begin to define the specific proteins involved in hemin binding and transport in *P. gingivalis*.

In *E. coli*, *N. gonorrhoeae*, *N. meningitidis*, *S. flexneri*, *V. cholerae*, *A. salmonicida*, and *Y. enterocolitica*, the ability to bind CR is induced by iron restriction (14, 27, 41, 47, 48, 50, 59, 61). A relationship between CR and hemin binding also exists in *S. flexneri*, *A. salmonicida*, and *Y. pestis* (13, 41, 47). The ability of *Yersinia* species and *S. flexneri* to absorb CR as well as hemin has also been correlated with virulence (13, 41, 47). A Cr^+ phenotype appears to enhance the ability of *S. flexneri* to invade and infect HeLa cells (11, 59), and Pgm^- mutants of *Y. pestis* which do not bind CR are avirulent (26, 63). Fetherston et al. (15) have shown that the *pgm* locus spans 102 kb of chromosomal DNA, all of which is deleted in the Pgm^- mutants of *Y. pestis*.

We have shown previously that a relationship exists between CR and hemin uptake in *B. thetaiotaomicron* and that hemin uptake is induced by growth under hemin-replete conditions (19). In agreement with these results, the data presented here also indicate that CR binding by *P. gingivalis* is induced by growth under hemin-replete conditions. Our findings also suggest that hemin and CR binding in *P. gingivalis* may be closely linked. Hemin induction of both CR and hemin binding in *P. gingivalis* is novel, and only a few reports have documented such a phenomenon (7, 55). In one study, Carman et al. (7) demonstrated that *P. gingivalis* whole cells and outer membranes bound significant amounts of hemin when bacteria had been grown in complex media supplemented with hemin. Cells grown in complex media without supplemental hemin still bound hemin; however, they bound much less than those grown with hemin. Although interpretation of these results is complicated because of the complex media employed by these investigators, the results support the concept that hemin binding by *P. gingivalis* is hemin inducible. Smalley et al. (55) have also reported that growth under hemin-excess conditions resulted in increased hemin-binding capacity of *P. gingivalis* extracellular vesicle preparations, whole cells, and EDTA-extracted outer membranes. These findings (7, 55) are in agreement with the results presented here and, taken together, suggest that in *P. gingivalis* hemin may induce the production of new outer membrane proteins that may function in hemin binding and/or transport.

In humans, hemin is typically found intracellularly and becomes available to extracellular pathogenic organisms only following its intracellular release. The mechanisms involved in hemin uptake are not precisely known; however, the first step is believed to be hemin binding to the cell surface. The ability to bind hemin has been described for *H. influenzae*, *Actinobacillus pleuropneumoniae*, *A. salmonicida*, *Y. pestis*, *S. flexneri*, and enteroinvasive *E. coli* (10, 13, 27, 45, 46, 59). In these organisms, hemin binding is typically induced by growth under iron-replete conditions. In *H. influenzae*, heme transport may involve one or several of the heme-repressible outer membrane proteins. One such heme-repressible protein has been shown to bind hemin and may be involved in the initial stages of hemin assimilation in *H. influenzae* (9, 10). This *H. influenzae* hemin-binding lipoprotein has recently been cloned and sequenced and has been shown to bind hemin in vitro and also to promote binding by *E. coli* recombinants expressing the protein (9). In *H. influenzae*, hemin is transported as a whole molecule and does not appear to serve simply as a carrier of iron into the cell in a mechanism analogous to a siderophore (10). *H. influenzae* can utilize protoporphyrin IX for growth,

and a specific enzyme appears to be responsible for the coordination of iron to protoporphyrin IX. The ability of *P. gingivalis* to grow with protoporphyrin IX in the presence of Fe suggests that like *H. influenzae*, *P. gingivalis* may also possess a ferrocyclase that functions in the coordination of iron.

In *Y. pestis*, adsorbed hemin is stored in the outer membrane during growth at 26°C but not at 37°C (61). Pendrak and Perry (42) have described the characterization of spontaneous avirulent Pgm⁻ mutants of *Y. pestis* that are defective in hemin storage but not in hemin utilization. These investigators have proposed the existence of two separate hemin loci that control (i) a hemin uptake component and (ii) hemin storage loci (42, 45). The hemin storage locus is found within a larger pigmented locus that seems to control both hemin and CR binding. Pendrak and Perry (43) have recently cloned and mapped structural genes for four proteins encoded on the hemin storage (*hms*) locus of *Y. pestis*. Three of the identified *hms* gene products, *Hms H*, -F, and -R, are required for an Hms⁺ phenotype in *Y. pestis*. Initial studies of the *Y. enterocolitica* (57) hemin transport system indicate that the entire hemin molecule is transported into the cell. Recently, the hemin receptor of *Y. enterocolitica* was identified, and its gene was cloned and sequenced (57). Hemin uptake in *Y. enterocolitica* was shown to be TonB dependent, similarly to other siderophore and vitamin B₁₂ uptake systems. In *V. cholerae*, at least two high-affinity systems for acquiring iron have been identified; a siderophore-mediated system and a hemin and hemoglobin utilization system. Henderson and Payne (24) have recently cloned several genes for *V. cholerae* hemin utilization and have shown that these are sufficient for the transport of hemin into the cell.

The ability to store hemin may help bacteria to obtain hemin despite the host's hemin-scavenging strategies. The hemin uptake system may represent an important survival mechanism especially for a mucosal pathogen like *P. gingivalis*, since a large amount of hemin is present at mucosal sites because of the desquamation of epithelial cells. In an ecosystem in which the levels of hemin are variable, the ability to store hemin becomes extremely important to the ultimate survival of *P. gingivalis*. Thus, when hemin levels in the gingival crevice rise, hemin can be stored for later use under conditions of hemin limitation. The ability to utilize and store hemin compounds may thus provide this pathogen with an alternative nutritional source of iron that is typically more abundant in humans.

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